Unique Features of Naive CD8$^+$ T Cell Activation by IL-2

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IL-2 has a pervasive influence on the immune system and dictates the survival and differentiation of multiple T cell subsets, including CD4 regulatory T cells, CD4 Th cells, and CD8 memory cells. IL-2 is synthesized by T cells during the early stages of the immune response and promotes T cell expansion and effector cell generation after initial activation via TCR signaling. Based on studies with activated T cell lines maintained in vitro, IL-2 is known to activate multiple signaling pathways that show considerable overlap with the pathways elicited via the TCR. In this paper, we have examined IL-2 signaling under TCR-independent conditions, namely by culturing purified resting naive CD8 T cells with IL-2 in the absence of Ag or APC. Under these conditions, we show in this study that IL-2 elicits a unique pattern of signaling associated with strong lymphocyte-specific protein tyrosine kinase/JAK3-dependent activation of the PI3K/AKT pathway with little or no involvement of STAT5, NF-κB, or the calcineurin/NFAT pathways. Such signaling induces marked proliferation associated with rapid and selective expression of eomesodermin but not T-bet and differentiation into long-lived central memory cells after adoptive transfer. ***The Journal of Immunology*****, 2013, 191: 5559–5573.**

Interleukin-2 is a potent growth factor for T cells and has both positive and negative effects on the immune response (1). Through its capacity to augment T cell responses in vitro, IL-2 was originally viewed as being crucial for the expansion and survival of Ag-reactive T cells, both for CD4 and CD8 T cells. With the discovery of Foxp3$^+$ CD4 regulatory T cells, however, the primary role of IL-2 may be to maintain the survival of regulatory T cells, thereby maintaining self-tolerance. However, many other cells express receptors for IL-2 (IL-2R), thus fostering the notion that IL-2 has positive effects on the immune response. Nevertheless, typical immune responses of T cells in vivo are largely intact in the absence of IL-2 (2, 3), which is surprising because activated CD4 and CD8 T cells synthesize IL-2 and express a high density of IL-2R. Despite this finding, it is now agreed that optimal immune responses in vivo do require IL-2, especially for the generation of CD8 memory T cells (4, 5). In addition, IL-2 has important qualitative effects on CD4 Th differentiation. Thus, together with TCR signals, high concentrations of IL-2 induce strong induction of particular transcription factors, notably T-bet and B lymphocyte–induced maturation protein 1 (Blimp-1), plus altered sensitivity to certain cytokines such as IL-6 and IL-12; these effects promote the generation of short-lived effector T cells rather than memory T cells and favor production of Th1 cells rather than Th17 cells or T follicular helper cells (6–9).

T cell stimulation by IL-2 is generally subsidiary to activation via the TCR. TCR activation involves a complex series of intracellular signaling events initiated by TCR recognition of specific peptide–MHC complexes on APC (10, 11). Modulated by the activity of CD45 (12), T cell signaling is initiated by phosphorylation of p56^{lck} and other Src family tyrosine kinases, which lead to activation of TCR/CD3 complexes, followed by recruitment and activation of Zap-70 and a series of downstream adaptor or scaffold proteins. Thereafter, activation of protein kinase Cθ (PKCθ) and the MAPK pathways, including p38, ERK, and JNK, cause gene transcription via NF-κB translocation to the nucleus. In parallel, intracellular release of Ca^{2+} leads to release of the phosphatase calcineurin, which in turn causes nuclear translocation of NFAT followed by synthesis of IL-2. These and other signaling events, notably activation of the PI3K/AKT pathway, lead to cell activation and entry into the cell cycle. T cell activation is associated with early synthesis of IL-2 and upregulation of CD25 (IL-2Rα) (i.e., the component of the αβγ IL-2R that controls high-affinity binding of IL-2). Hence, the combined synthesis of IL-2 and IL-2Rs plays a key role in regulating the intensity of initial T cell expansion and the differentiation of the responding T cells into subsets of effector and memory T cells.

Although TCR-induced activation and expansion of T cells are presumed to reflect a combination of TCR/CD3 and IL-2/IL-2R signaling, the pathways involved in IL-2 signaling are unclear. Most of the information on IL-2 signaling has come from studies on cell lines or activated T cells, and these studies have implicated the involvement of the JAK3/STAT5, MAPK/ERK, and PI3K/AKT

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Received for publication August 27, 2013. Accepted for publication September 19, 2013.

This work was supported by project and program grants (to J.-H.C. and J.S., respectively) funded by the National Health and Medical Research Council of Australia.

J.-H.C. and H.-O.K. designed and performed the majority of the experiments, coordinated the study, analyzed and interpreted the data, and wrote the manuscript. K.-S.K., D.-H.Y., and C.D.S. provided supporting information. J.S. coordinated the study, analyzed and interpreted the data, and wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AKTi, AKT inhibitor IV; B6, C57BL/6; Bcl-6, B cell lymphoma 6; BHI, brain–heart infusion; Blimp-1, B lymphocyte–induced maturation protein 1; CsA, cyclosporin A; Eomes, eomesodermin; ICS, intracellular cytokine staining; IL-2R, IL-2 receptor; IRF4, IFN regulatory factor 4; JAK3i, JAK3 inhibitor VI; LCK, lymphocyte-specific protein tyrosine kinase; Lm-gp33, recombinant *Listeria monocytogenes* strains that express the gp33–41 epitope; Lm-ova, recombinant *Listeria monocytogenes* strains that express OVA; LN, lymph node; LY, LY294002; MHC-I, MHC class I; MP, memory-phenotype; mTOR, mammalian target of rapamycin; OVAp, OVA peptide; PKCθ, protein kinase Cθ; Rapa, rapamycin; sAPC, splenic APC; SB, SB203580; SOCS, suppressor of cytokine signaling; SP, SP600125; WT, wild-type.

pathways in IL-2 signaling (13–15). However, the activated status of the cells tested raises the question whether stimulation via IL-2 requires joint TCR/CD3 signaling. Whether naive T cells can be stimulated by IL-2 in the absence of TCR signals remains an important unanswered question.

Because CD25 expression requires T cell activation, IL-2R expression on resting naive T cells is limited to the low-affinity βγ IL-2R. For CD8 T cells, IL-2Rβ (CD122) expression is prominent on resting central memory cells ($CD44^{hi}CD62L^{hi}$ cells), but is also found at a significant level on typical naive ($CD44^{lo}CD62L^{hi}$) cells, though only on CD8 and not CD4 T cells (16). Recent work has shown that IL-2Rβγ on naive CD8 T cells is functional and allows the cells to respond vigorously to moderate concentrations of IL-2 (or IL-15) as manifested by proliferation and differentiation into effector cells, both in vitro and in vivo (17, 18). For in vitro responses, IL-2–induced proliferation applies to naive ($CD44^{lo}$) cells in the absence of APC, implying a lack of requirement for TCR/MHC class I (MHC-I) interaction on APC or contact with APC-derived cytokines. Nevertheless, IL-2 stimulation is not totally TCR independent because naive CD8 T cells designed to lack MHC-I molecules give reduced proliferative responses to IL-2, both in vitro and in vivo (17, 18). Hence, IL-2 responsiveness is partly dependent on the low-level TCR signals that result when CD8 T cells contact self–MHC-I ligands on various cell types, including neighboring CD8 T cells, in vivo.

With regard to physiological significance, stimulation of naive T cells by γ_c cytokines plays an important role in T cell homeostasis (19). Thus, T cell contact with IL-7 and also IL-15 in normal mice is crucial for maintaining long-term survival of T cells in interphase. In addition, naive CD8 T cells undergo marked proliferation and expansion following exposure to elevated levels of IL-7 in lymphopenic mice or raised levels of IL-2 and/or IL-15 in IL-2R–deficient *il2ra*$^{-/-}$ and *il2rb*$^{-/-}$ hosts (17). Likewise, therapy with IL-7 and IL-2 is used to expand T cells in lymphopenic patients (20, 21). Yet virtually nothing is known about the signaling pathways involved in these Ag-independent proliferative responses. In this paper, using IL-2 as a model, we have compared the signaling pathways that control stimulation of purified naive CD8 T cells by IL-2 alone versus TCR/CD3 ligation. The results reveal that, despite some overlap, the signaling pathways elicited by these two forms of stimulation are strikingly different.

Materials and Methods

Mice

C57BL/6 (B6) and B6.SJL (Ly5.1) mice were purchased from the Animal Resources Centre. *Tap1*$^{-/-}$ and *jak3*$^{-/-}$ (all on a B6 background) mice were purchased from The Jackson Laboratory. The *jak3*$^{-/-}$ mice were crossed with B6 to obtain *jak3*$^{+/-}$ mice. The *jak3*$^{+/-}$ mice were then bred to obtain *jak3*$^{+/+}$, *jak3*$^{+/-}$, and *jak3*$^{-/-}$ littermates. Sources of *cnab*$^{-/-}$ (22) mice as well as OT-I.Thy1.1, OT-I.Ly5.1, 2C, 2C.Thy1.1, and 2C. *il2ra*$^{-/-}$ TCR-transgenic mice, all on a B6 background, were described previously (17, 23). *Stat5*$^{-/-}$ (24) and *pkcθ*$^{-/-}$ (25) mice (all on a B6 background) were originated by Dr. J. N. Ihle (St. Jude Children's Research Hospital) and Dr. D. R. Littman (Skirball Institute), respectively. All mice were maintained under specific pathogen-free conditions and used at 6–12 wk of age, according to protocols approved by the Animal Experimental Ethics Committee at the Garvan Institute.

Reagents

Recombinant mouse IL-2, IL-4, IL-7, IL-12, IL-15, and IL-21 were all purchased from PeproTech. Peptides (SIYRYYGL, SIYRp; and SIINFEKL, OVA peptide [OVAp]) were purchased from Mimotopes. LY294002 (LY), AKT inhibitor IV (AKTi), PP2, JAK3 inhibitor VI (JAK3i), U0126, PD98059, SB203580 (SB), SP600125 (SP), rapamycin (Rapa), cyclosporin A (CsA), and VIVIT were purchased from Calbiochem and Sigma-Aldrich and dissolved in DMSO (Sigma-Aldrich).

Abs and flow cytometry

Cell suspensions were prepared and stained for FACS analysis of cell-surface markers using PBS containing 2% FBS and 0.05% sodium azide with the following fluorochrome-conjugated Abs to (from BD Biosciences and eBioscience): CD8α (53-6.7), CD25 (7D4), CD27 (LG.7F9), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD122 (TM-β1), CD127 (A7R34), CCR7 (4B12), Ly6C (HK1.4), CD45.1 (A20), and CD90.1 (HIS51). Flow cytometry samples were run using an LSR II or FACSCanto II (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Intracellular staining

For intracellular cytokine staining (ICS), cells stimulated with indicated stimuli in the presence of GolgiStop (BD Biosciences) were stained for cell-surface markers, fixed, and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and then stained with fluorochrome-conjugated Abs to IFN-γ (XMG1.2) and TNF-α (MP6-XT22) using Perm/Wash buffer (BD Biosciences). The same ICS protocol was used for analyzing granzyme B expression with fluorochrome-conjugated Ab to granzyme B (GB11; Caltag Laboratories). Intracellular staining for T-bet and eomesodermin (Eomes) expression was performed with Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions using fluorochrome-conjugated Abs to T-bet (4B10) and Eomes (Dan11mag; all from eBioscience).

T cell purification and in vitro stimulation

Pooled lymph node (LN) cells from mice indicated were stained with fluorochrome-conjugated Abs to CD4, CD8, CD25, CD44, and CD62L and sorted to obtain naive $CD44^{lo}CD62L^{hi}CD8^{+}$ or $CD44^{lo}CD62L^{hi}CD25^{-}$ $CD4^{+}$ T cells using an FACSAria (BD Biosciences). Purity was routinely tested after cell sorting and was >99%. For in vivo transfer experiments, naive 2C or OT-I TCR-transgenic $CD8^{+}$ T cells were purified by a negative selection using MACS with a $CD8^{+}$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions with a minor modification of inclusion of pretitrated amounts of biotin-conjugated anti-CD44 Ab to deplete $CD44^{hi}$ memory-phenotype (MP) $CD8^{+}$ T cells. For in vitro stimulation, sorted naive CD8 T cells ($0.5–2 \times 10^5$ cells/well in 96-well plates) were cultured with IL-2 or other γ_c cytokines indicated (all 1 μg/ml unless otherwise described) or plate-bound anti-CD3 Ab (145-2C11; 2.5 μg/ml unless otherwise described) ± anti-CD28 Ab (37.51; 5 μg/ml) or IL-2 (10 ng/ml). In some cultures with IL-2 (1 μg/ml), cells were supplemented either with cytokines IL-12 or IL-21 (20 ng/ml) or with anti-CD3 Ab as a soluble (0.01–10 μg/ml) or plate-bound form (2.5 μg/ml) or with T cell–depleted irradiated (2000 cGy) B6 splenic APC (sAPC; 1×10^6 cells) ± soluble anti-CD3 Ab (0.1 μg/ml). In coligation experiments, cells labeled with CFSE were stimulated with IL-2 (1 μg/ml) or plate-bound anti-CD3 Ab (2.5 μg/ml) ± 10 μg/ml plate-bound anti-CD5 (53-7.3; eBioscience) or anti-CD45 Ab (30-F11; eBioscience). For inhibitor experiments, purified naive CD8 T cells were preincubated with various chemical inhibitors for 15–30 min before in vitro culture with stimuli indicated; unless otherwise described, LY, U1206, SB, SP, AKTi, Rapa, VIVIT, and PP2 inhibitors were used at a concentration of 2, 5, 10, 10, 0.5, 0.1, 2, and 2 μM, respectively, and as a control, PBS containing 0.1–0.2% DMSO was used.

Proliferation assay

At various time points after in vitro culture with the indicated stimuli, cells were added with [^{3}H]thymidine (1 μCi/well) and, after a 6–12-h pulse, harvested, and measured by a β-counter (TopCount Microplate Scintillation Counter; PerkinElmer). For visualization of cell division, purified naive CD8 T cells were labeled with CFSE (Invitrogen) and analyzed by flow cytometry.

Immunoblot analysis and immunoprecipitation

Cells were washed with ice-cold PBS and lysed on ice for 15–30 min in a lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml aprotinin and leupeptin). Cell lysates were resolved by 4–12% Bis-Tris SDS-PAGE Gel (Invitrogen), transferred onto nitrocellulose membrane (Invitrogen), blocked with 5% dry nonfat milk in TBS (pH 7.4) containing 0.1% Tween-20, and probed with the following Abs to (from Cell Signaling Technology unless otherwise described): p-ERK1/2 (Thr^{202}/Tyr^{204}; D13.14.4E), ERK1/2 (Santa Cruz Biotechnology), p-AKT (Ser^{473}; 193H12), AKT, p-p38 ($Thr^{180/182}$; D3F9), p38, p-JNK ($Thr^{183/185}$; 81E11), JNK (Santa Cruz Biotechnology), p–mammalian target of rapamycin (mTOR; Ser^{2448};

D9C2), p-JAK3 (Tyr$^{980/981}$; D44E3), JAK3 (Abcam), p-JAK1 (Tyr$^{1022/1023}$), p-STAT5A/B (Tyr$^{694/699}$; A11W; Millipore), STAT5, p-STAT3 (Tyr^{705}; D3A7), STAT3 (79D7), p-Tyrosine (4G10; Millipore), cyclin D3 (D-7; Santa Cruz Biotechnology), cyclin D2 (M-20; Santa Cruz Biotechnology), p21 (F-5; Santa Cruz Biotechnology), p-S6 Ribosomal Protein (Ser$^{235/236}$; D57.2.2E), p-4E,BP1 (Thr$^{37/46}$; 236B4), p-PI3K p85 (Tyr^{458})/p55 (Tyr^{199}), PI3K p85 (19H8), p-p56^{lck} (Tyr^{394}; Sigma-Aldrich and Santa Cruz Biotechnology), p56^{lck} (3A5; Santa Cruz Biotechnology), T-bet (4B10; Santa Cruz Biotechnology), Eomes (21Mags8; eBioscience), Blimp-1 (6D3; Santa Cruz Biotechnology), B cell lymphoma 6 (Bcl-6), Bmi1 (1.T.21; Abcam), IFN regulatory factor 4 (IRF4; P173), suppressor of cytokine signaling (SOCS) 1 (C-20; Santa Cruz Biotechnology), and β-actin (AC-15, AC-74; Sigma-Aldrich). Immunoreactivity was detected by ECL detection system according to the manufacturer's instructions (GE Healthcare). For immunoprecipitation, cell lysates were incubated with Abs to p56^{lck} (3A5), IL-2Rβ (M-20 [Santa Cruz Biotechnology] and TM-β1 [BD Biosciences]), or Shc (Abcam), followed by Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). The immunoprecipitated complexes were prepared and immunoblotted with indicated Abs described above.

Confocal staining

For confocal staining, cells cultured with indicated stimuli were washed, placed at 0.5–1 × 10^5 cells on a poly-L-lysine–coated glass slide (Sigma-Aldrich), and allowed to adhere to the slide for 5 min at room temperature. The cells were washed, fixed for 20 min with cold 4% paraformaldehyde in PBS, permeabilized for 5 min with 0.1% Triton X-100 in PBS, and then blocked for 15 min with 5% normal goat serum in PBS containing 1% BSA. Cells were stained for 45 min with a primary Ab to NFATc1 (7A6; Santa Cruz Biotechnology) or NF-κB p65 (C-20; Santa Cruz Biotechnology), washed, blocked, and then reincubated for 30 min with Alexa Fluor 488–conjugated anti-mouse or anti-rabbit IgG (Invitrogen). In some experiments, the stained cells were further incubated for 5 min with DAPI (Invitrogen). The final slides were washed with PBS and mounted in ProLong Gold Antifade Reagent (Invitrogen) and analyzed using a Leica TCS2 laser scanning confocal microscope (Leica Microsystems) for acquiring fluorescence images.

Cytokine ELISA and Th1/Th2 polarization

For detection of IFN-γ secretion, culture supernatants from cells incubated with indicated stimuli were collected and analyzed by a standard protocol using a cytokine sandwich ELISA kit for IFN-γ (BD Biosciences) according to the manufacturer's instructions. For generating Th1/Th2-polarized cells, naive B6 CD4 and CD8 T cells were stimulated with 1 μg/ml IL-2 (5 d; CD8 T cells only) or plate-bound anti-CD3 (2.5 μg/ml) plus anti-CD28 (5 μg/ml) Abs (3 d; both CD4 and CD8 T cells) in the presence of Th1- (10 ng/ml IL-12 plus 10 μg/ml anti–IL-4; 11B11, BD Biosciences), Th2- (10 ng/ml IL-4 plus 10 μg/ml anti–IFN-γ; XMG1.4, BD Biosciences), or Th0-polarizing (none) conditions. Cells were then harvested, washed, and recultured with plate-bound anti-CD3 Ab (2.5 μg/ml). At 1 d after restimulation, culture supernatants were collected and analyzed by ELISA using a kit for detecting IL-2, IFN-γ, IL-4, IL-6, and IL-10 (all from BD Biosciences).

Adoptive transfer and in vivo stimulation

For donor cell survival experiments, MACS-purified naive 2C or OT-I CD8 T cells (Thy1.1) were cultured for indicated times (2 to 3 d for plate-bound anti-CD3 and 5 d for IL-2 stimulation) by the aforementioned in vitro stimulation conditions and then transferred i.v. to normal B6 mice (0.5–2 × 10^6 cells/mouse; n = 5–8/group). At various time points after adoptive transfer, PBL, spleen, and/or LN cells were analyzed for donor cell recovery and expression of surface markers by flow cytometry; in some experiments, cells were restimulated for 5 h with 0.1 μM SIYRp (for 2C donor cells) or OVAp (for OT-I donor cells) and analyzed for intracellular IFN-γ and TNF-α production from donor cells using the ICS protocol described above. For in vivo regulation of T-bet and Eomes, MACS-purified OT-I CD8 T cells (Thy1.1) were stimulated with indicated stimuli and transferred i.v. to normal B6 mice (1 × 10^6 cells/mouse; n = 5/group). At 56–78 d after adoptive transfer, spleen cells were analyzed for T-bet and Eomes expression using intracellular staining described above. For T-bet and Eomes expression with direct in vivo stimulation, MACS-purified 2C or OT-I CD8 T cells (Thy1.1) were transferred i.v. to irradiated (550 cGy) B6 or *tap1*$^{-/-}$ mice (1 × 10^6 cells/mouse; n = 3 to 4/group) and injected i.p. with either IL-2 (1 μg)/anti–IL-2 (S4B6; 10 μg) complexes (26) or 10 μM SIYRp (for 2C cells); in some experiments, mice were injected i.p. with IL-2/anti–IL-2 complexes and 10 μM OVAp (for OT-I cells). At 6–42 d after adoptive transfer, spleen cells were analyzed for T-bet and Eomes expression by flow cytometry.

In vivo CTL assay

At various indicated time points, recipient B6 mice injected with 2C or OT-I CD8 T cells stimulated in vitro with indicated stimuli were transferred i.v. with an equal mixture of CFSE^{hi}- (2 μM)- and CFSE^{lo}-labeled (0.2 μM) B6 (Ly5.1) splenocytes (5 × 10^6 cells/mouse; n = 2 to 3/group). SIYRp (2 μM; for 2C donor cells) or OVAp (for OT-I donor cells) was pulsed only on the CFSE^{hi} population. At 1 d after transfer of CFSE-labeled unpulsed and peptide-pulsed spleen cells, spleen and LN cells were analyzed, and the proportions of CFSE^{lo} and CFSE^{hi} cells were measured by flow cytometry.

Bacterial infection

Recombinant *Listeria monocytogenes* strains that express OVA (Lm-ova) and the gp33–41 epitope (Lm-gp33) of lymphocytic choriomeningitis virus, respectively, were originated by Dr. H. Shen (University of Pennsylvania) and grown in brain–heart infusion (BHI) medium (BD Biosciences) supplemented with 5 mg/ml erythromycin. At midlog phase (OD_{600} = 1), bacteria were harvested, frozen (in 20% glycerol), and stored at −80°C. CFUs were determined by performing serial dilutions on BHI agar plates. For bacterial infection, MACS-purified naive OT-I CD8$^+$ cells (Ly5.1) were first cultured with either IL-2 (1 μg/ml; 5 d) or plate-bound anti-CD3 (2.5 μg/ml; 2 d) ± anti-CD28 (5 μg/ml), washed, and transferred i.v. to normal B6 mice; mice either uninjected or injected with naive OT-I cells freshly isolated were used as a control (2 × 10^6 cells/mouse). For the expansion experiment, at 1 d after adoptive transfer, mice were infected i.v. with Lm-ova or Lm-gp33 (2 × 10^4 CFU/mouse; n = 5/each), and at 3 d postinfection, spleen cells were analyzed for donor cell recovery by flow cytometry. Viable bacterial counts were determined by homogenizing spleen from infected mice in PBS containing 0.05% Triton X-100 and plating 10-fold dilutions on BHI agar plates. For the protection experiment, at 3 d after OT-I cell transfer, mice were infected i.v. with Lm-ova (2 × 10^5 CFU/mouse; n = 6–8/group), and survival kinetics were measured at indicated time points postinfection.

Statistical analysis

The unpaired two-tailed Student t test was used to determine statistically significant differences. The p values < 0.05 were considered statistically significant.

Results

PI3K-dependent signaling of naive CD8 T cells by IL-2

As shown previously (18), CFSE-labeled purified naive (CD44^{lo}) CD8 T cells from normal B6 or 2C TCR-transgenic mice proliferated extensively when cultured with high concentrations of IL-2 without APC (Fig. 1A, 1B). Proliferation was associated with upregulation of CD25, CD44, and other activation markers (Fig. 1A, 1B, Supplemental Fig. 1A) and was marked with both CD25-deficient (*il2ra*$^{-/-}$) and wild-type (WT) CD8 cells (Fig. 1B, Supplemental Fig. 1B), indicating signaling via the low-affinity βγ IL-2R. IL-2 also induced phosphorylation of ERK and AKT, although less rapidly than with anti-CD3 ligation (Fig. 1C); similar results applied to IL-15, but other γ_c cytokines were ineffective (Fig. 1D). In contrast to stimulation with cross-linked anti-CD3 Ab, IL-2–driven proliferation was largely resistant to U0126, an ERK inhibitor (Fig. 1E), but was strongly blocked by LY and AKTi, which inhibit PI3K and downstream AKT, respectively (Fig. 1F, 1G). Like CD3 ligation, IL-2 also induced phosphorylation of other MAPKs, such as p38 and JNK (Fig. 1H). However, blockade of these latter pathways with SB (for p38) and SP (for JNK) caused only slight inhibition of proliferation and CFSE division (Fig. 1I, 1J), indicating only minor dependency on p38 and JNK.

These findings indicated that IL-2 stimulation was largely ERK independent but strongly PI3K/AKT dependent. Like CD3 ligation, IL-2 also induced phosphorylation of mTOR, a known target of AKT (Fig. 1H). Moreover, addition of rapamycin, an mTOR inhibitor, markedly inhibited proliferation induced by IL-2, as well as by cross-linked anti-CD3 Ab (Fig. 1K). Hence, IL-2–induced proliferation of naive CD8 cells depended far more on the PI3K/

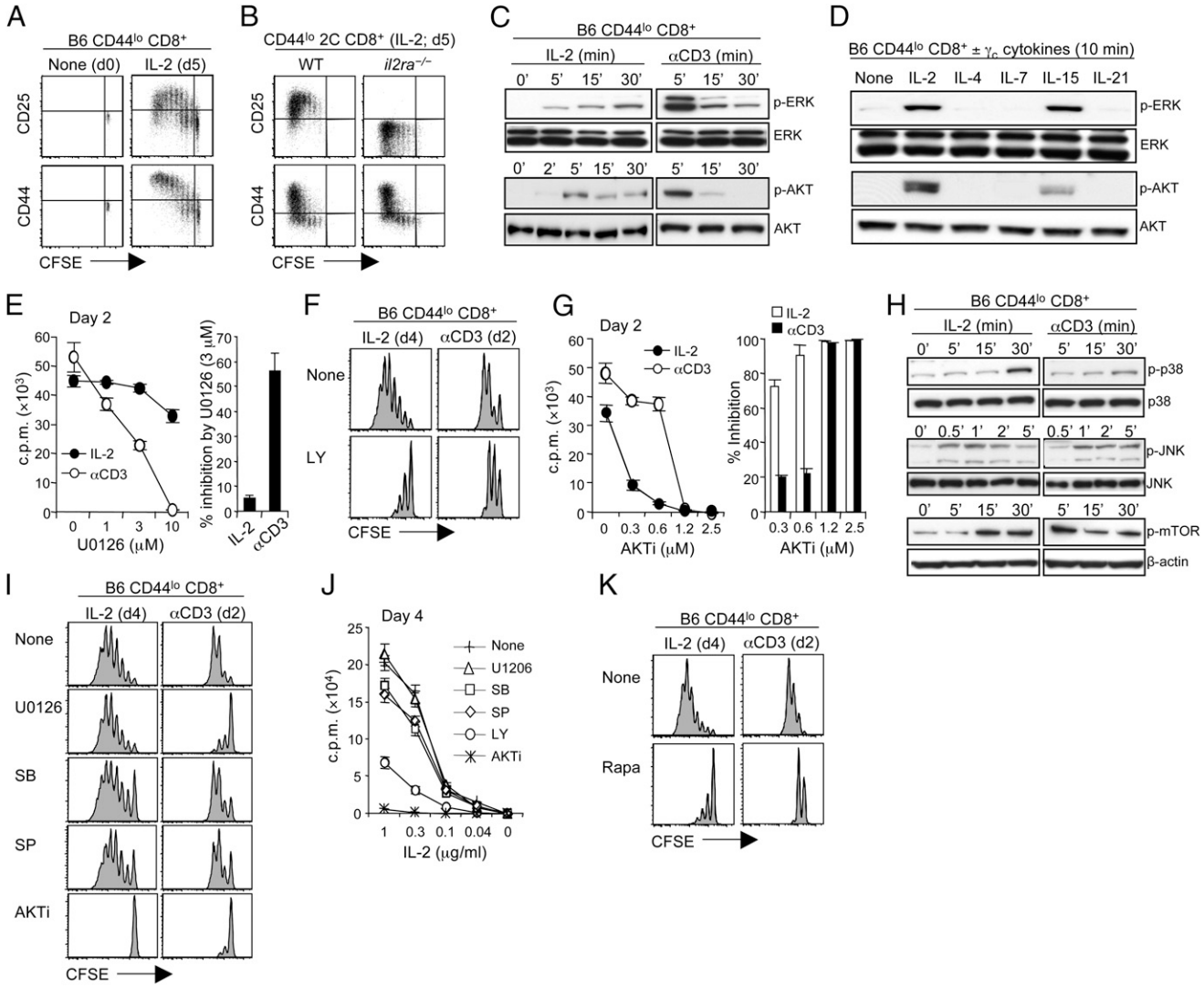

FIGURE 1. Proliferation by IL-2 requires PI3K/AKT/mTOR but not ERK pathway. CFSE division and expression of CD25 and CD44 on naive CD8 cells from B6 (**A**) or *il2ra*$^{-/-}$ and WT 2C mice (**B**) after 5-d IL-2 culture. Phosphorylation of ERK and AKT after incubating naive B6 CD8 cells for indicated times with IL-2 or plate-bound anti-CD3 (**C**) or indicated γ_c cytokines (**D**). (**E**) Proliferation of naive B6 CD8 cells after 2 d culture with IL-2 or plate-bound anti-CD3 with U0126. Percent inhibition of proliferation at a concentration of 3 μM relative to no inhibitor is shown. (**F**) CFSE division of naive B6 CD8 cells after 2–4 d culture with IL-2 or plate-bound anti-CD3 ± LY. (**G**) Naive B6 CD8 cells were cultured as in (E) with AKTi. Proliferation and percent inhibition are shown. (**H**) Phosphorylation of p38, JNK, and mTOR analyzed as in (C). (**I**) CFSE division of naive B6 CD8 cells after 2–4 d culture with IL-2 or plate-bound anti-CD3 ± U1206, SB, SP, or AKTi. (**J**) Proliferative responses of naive B6 CD8 cells after 4-d IL-2 culture with indicated inhibitors. (**K**) CFSE division of naive B6 CD8 cells after 2–4 d culture with IL-2 or plate-bound anti-CD3 ± Rapa. Data are representative of three independent experiments and shown as mean ± SD. α, Anti.

AKT pathway than the MAPK pathways. By contrast, CD3-induced proliferation was more dependent upon the MAPK pathways than PI3K/AKT (compare Fig. 1E with Fig. 1G).

Role of the JAK/STAT pathway: dependency on JAK3 but not STAT5

Consistent with the role of the JAK3/STAT5 pathway in activation via PI3K/AKT (27), stimulation of naive CD8 cells by IL-2 caused rapid phosphorylation of JAK3 and STAT5, with weaker phosphorylation of STAT3 (Fig. 2A, 2B); other γ_c cytokines had variable effects on these substrates. The JAK3/STAT5 pathway appeared to be obligatory for proliferation because addition of JAK3i abolished IL-2–induced proliferation (Fig. 2C); by contrast, JAK3i caused only mild inhibition of proliferation induced by CD3 or CD3/CD28 ligation (Fig. 2C and data not shown). Similar findings occurred in studies with JAK3-haploinsufficient CD8 T cells obtained from JAK3 heterozygous (*jak3*$^{+/-}$) mice (28). In this study, the striking finding was that proliferation and activation marker expression of *jak3*$^{+/-}$ naive CD8 cells were near normal with CD3 ligation but very low with IL-2 stimulation (Fig. 2D).

To examine the role of STAT5 in proliferation, we used T cells from combined STAT5a and STAT5b-deficient (*stat5*$^{-/-}$) mice (24). Notably, the capacity of IL-2 to augment proliferation induced by CD3 ligation was high with WT CD8 cells but very low with *stat5*$^{-/-}$ cells (Fig. 2E). In sharp contrast, the capacity of IL-2 alone to induce naive CD8 cells to proliferate and upregulate activation markers was as high with *stat5*$^{-/-}$ cells as with WT cells (Fig. 2F, 2G). These findings applied on day 3 of culture. However, on day 5, despite vigorous proliferation, *stat5*$^{-/-}$ cells showed a mild reduction in granzyme B synthesis (Fig. 2F, 2G). These findings suggest that for IL-2 stimulation, STAT5 is not required for proliferation but may be needed for later cell survival and differentiation into effector cells.

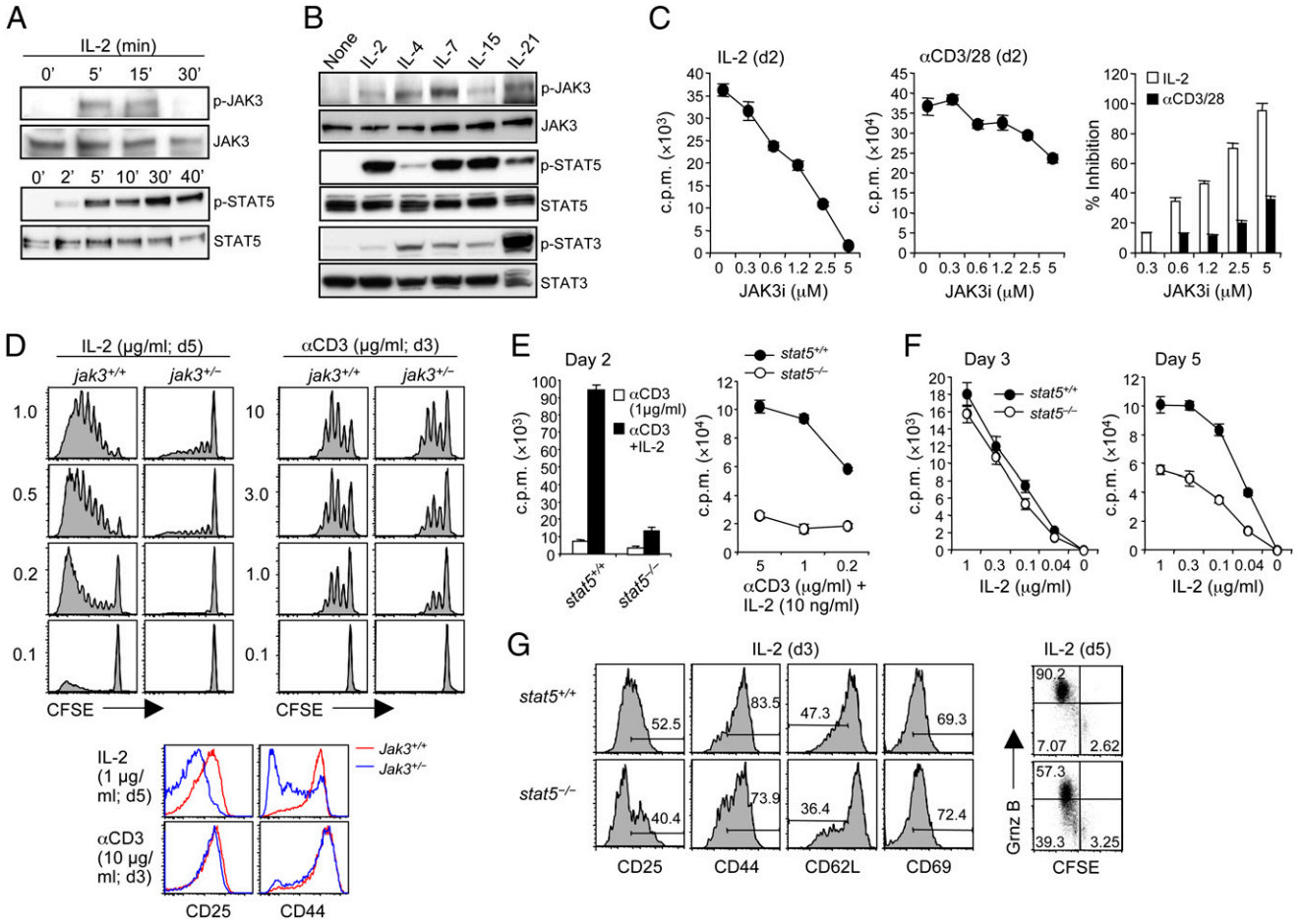

FIGURE 2. IL-2–driven proliferation is JAK3 dependent but STAT5 independent. Phosphorylation of JAK3 and STAT5 (**A**, **B**) and STAT3 (B) after culturing naive B6 CD8 cells for indicated times with IL-2 (A) or indicated γ_c cytokines (B). (**C**) Proliferation and percent inhibition of naive B6 CD8 cells after 2 d culture with IL-2 or plate-bound anti-CD3/CD28 with JAK3i. (**D**) CFSE division (*top panel*) and CD25 and CD44 expression (*bottom panel*) in WT and $jak3^{+/-}$ naive CD8 cells after 3–5 d culture with IL-2 or plate-bound anti-CD3. (**E**) Proliferation of WT and $stat5^{-/-}$ naive CD8 cells after 2 d stimulation with plate-bound anti-CD3 (1 μg/ml, *left panel*; 0.2–5 μg/ml, *right panel*) ± IL-2 (10 ng/ml). (**F**) Proliferation of WT and $stat5^{-/-}$ naive CD8 cells after 3–5 d IL-2 culture. (**G**) Expression of indicated activation markers (*left panel*; 3 d) and CFSE division and granzyme B (Grnz B) synthesis (*right panel*; 5 d) in cells cultured as in (E). Percentages of gated region are shown. Data are representative of at least two independent experiments and shown as mean ± SD. α, Anti.

Role of p56^{lck}

As for activated T cells (29, 30), stimulation of naive CD8 cells by IL-2 caused rapid tyrosine phosphorylation of p56^{lck} (lymphocyte-specific protein tyrosine kinase [LCK]; Figure 3A), and, as with CD3 ligation, proliferation induced by IL-2 was ablated by an LCK inhibitor, PP2 (Fig. 3B). Likewise, PP2 markedly inhibited IL-2–induced phosphorylation of AKT and ERK (Fig. 3C), but not of p38 or JNK (Fig. 3D), and led to decreased levels of cell cycle–promoting proteins cyclin D2, D3, and p21 (Fig. 3E); this effect was not seen with the ERK inhibitor U0126 as a control. Downstream of AKT, PP2 also inhibited IL-2–induced phosphorylation of mTOR as well as two further downstream mTOR targets, 40S ribosomal S6 and 4EBP1 (Fig. 3F). These findings are summarized in Fig. 3O.

As for AKT, PP2 inhibited phosphorylation of upstream PI3K (p85α subunit) (Fig. 3G), implying that IL-2/IL-2R signaling involves a close link between the LCK and PI3K/AKT pathways. Indeed, IL-2 exposure resulted in rapid LCK-dependent tyrosine phosphorylation of IL-2Rβ and its binding to several tyrosine-phosphorylated proteins (Fig. 3H, 3I). Notably, IL-2 induced the LCK-dependent association of IL-2Rβ with PI3K (Fig. 3J). These findings suggest that LCK couples IL-2Rβ signaling to PI3K/AKT. This interaction involved JAK3 because, in addition to ERK and AKT, PP2 blocked phosphorylation of JAK3, but not JAK1 (Fig. 3K). By contrast, PP2 failed to impair phosphorylation of STAT5 (Fig. 3K, 3L). Hence, for IL-2 stimulation, LCK was not required for STAT5 phosphorylation, yet was crucial for proliferation through activation of the PI3K/AKT pathway.

With regard to how LCK influences IL-2–induced ERK phosphorylation (Fig. 3C), it is of interest that PP2 blocked phosphorylation of Shc (Fig. 3M), an adaptor protein known to associate with IL-2Rβ and couple IL-2R signaling with the ERK pathway (31). However, whether such signaling involved JAK3 is questionable because phosphorylation of LCK, ERK, and STAT5 in response to IL-2 was as high with $jak3^{+/-}$ cells as with $jak3^{+/+}$ cells (Fig. 3N). These findings contrasted with the minimal capacity of $jak3^{+/-}$ cells to proliferate and phosphorylate AKT in response to IL-2 (Figs. 2D, 3N).

Collectively, the data indicate that LCK plays a crucial role in IL-2 signaling and involves two different downstream pathways, namely the JAK3-dependent PI3K/AKT/mTOR pathway, as well as a separate JAK3-independent pathway leading to ERK activation, perhaps via Shc (Fig. 3O). Only the former pathway was essential for proliferation.

Role of NF-κB and NFAT

As expected, subjecting naive CD8 cells to ligation with cross-linked anti-CD3 or anti-CD3/CD28 Abs led to a typical pattern of downstream TCR/CD3 signaling, namely rapid nuclear translocation of NFAT and p65 (NF-κB) (Fig. 4A, 4B). Proliferation

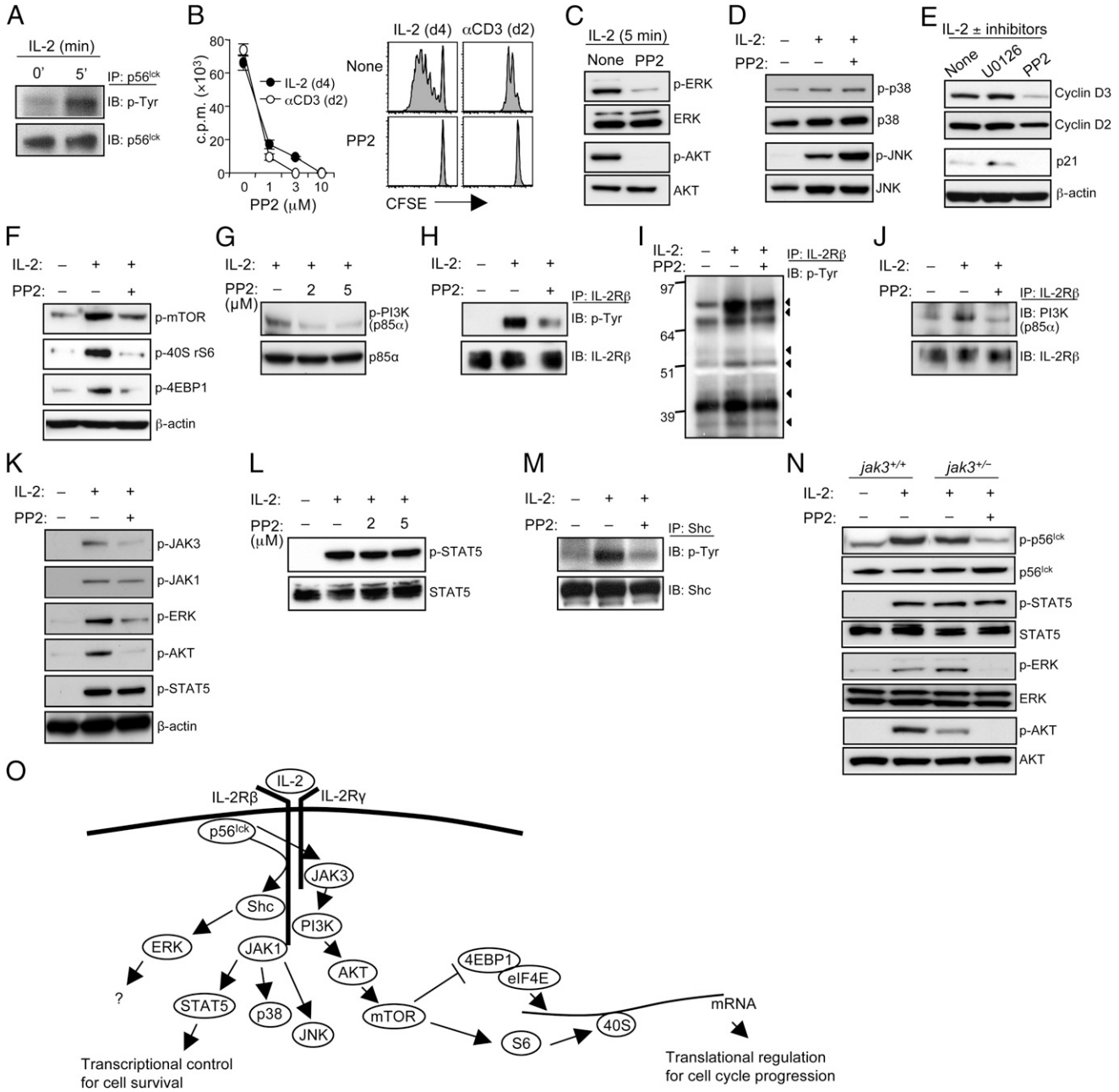

FIGURE 3. Role of p56^{lck} and JAK3 in IL-2 signaling. (**A**) Tyrosine phosphorylation of p56^{lck} after 5-min IL-2 exposure on naive B6 CD8 cells. (**B**) Proliferation (*left panel*) and CFSE division (*right panel*) of naive B6 CD8 cells after 2–4 d culture with IL-2 or plate-bound anti-CD3 with PP2. Phosphorylation of ERK and AKT (**C**) and p38 and JNK (**D**) after 1–5 min IL-2 exposure on naive B6 CD8 cells ± PP2. (**E**) Expression of indicated cell-cycle proteins after 2 d IL-2 exposure on naive B6 CD8 cells ± U0126 or PP2. (**F**) Phosphorylation of mTOR, 40S ribosomal S6 (rS6), and 4EBP1 after 20-min IL-2 exposure on naive B6 CD8 cells ± PP2. (**G**) Phosphorylation of p85α subunit of PI3K after 10-min IL-2 exposure on naive B6 CD8 cells with PP2. Tyrosine phosphorylation of IL-2Rβ (**H**) and association with multiple tyrosine-phosphorylated (p-Tyr) proteins (**I**) and PI3K (**J**) after 5-min IL-2 exposure on naive B6 CD8 cells ± PP2. Phosphorylation of JAK3, JAK1, ERK, AKT and STAT5 (**K**) and STAT5 (**L**) and Shc (**M**) at 5–10 min IL-2 exposure on naive B6 CD8 cells ± PP2. (**N**) Phosphorylation of p56^{lck}, STAT5, ERK, and AKT at 5–10 min IL-2 exposure on naive WT and *Jak3*$^{+/-}$ CD8 cells ± PP2. Data are representative of at least three independent experiments and shown as mean ± SD. (**O**) Proposed model of IL-2R signaling for naive CD8 T cells, outlining unique role of p56^{lck} in linking JAK3 to the PI3K/AKT/mTOR pathway independent of ERK and STAT5 to promote a priming signal for activation and proliferation. α, Anti; eIF4E, eukaryotic initiation factor 4E; IB, immunoblotting.

driven by CD3 ligation was heavily dependent on the calcineurin/NFAT pathway because proliferation and CD44 upregulation were blocked by addition of either an NFAT inhibitor, VIVIT, or a calcineurin inhibitor, CsA (Fig. 4C–E). For the NF-κB–linked pathway, signaling via PKCθ, which is known to be required for NF-κB activation (25), was crucial because proliferation and upregulation of activation markers were very low with *pkcθ*$^{-/-}$ CD8 cells (Fig. 4F, 4G).

In marked contrast to CD3 ligation, stimulating naive CD8 cells with IL-2 failed to induce nuclear translocation of NF-κB (Fig. 4A, 4B). In line with this finding, IL-2–induced proliferation and upregulation of activation markers were unimpaired in CD8 cells lacking PKCθ (Fig. 4F, 4G). In contrast to NF-κB, nuclear translocation of NFAT was clearly detectable after IL-2 stimulation, though only on day 3 of culture and not on day 1 (Fig. 4A, 4B). Notably, the late IL-2–induced nuclear translocation of NFAT

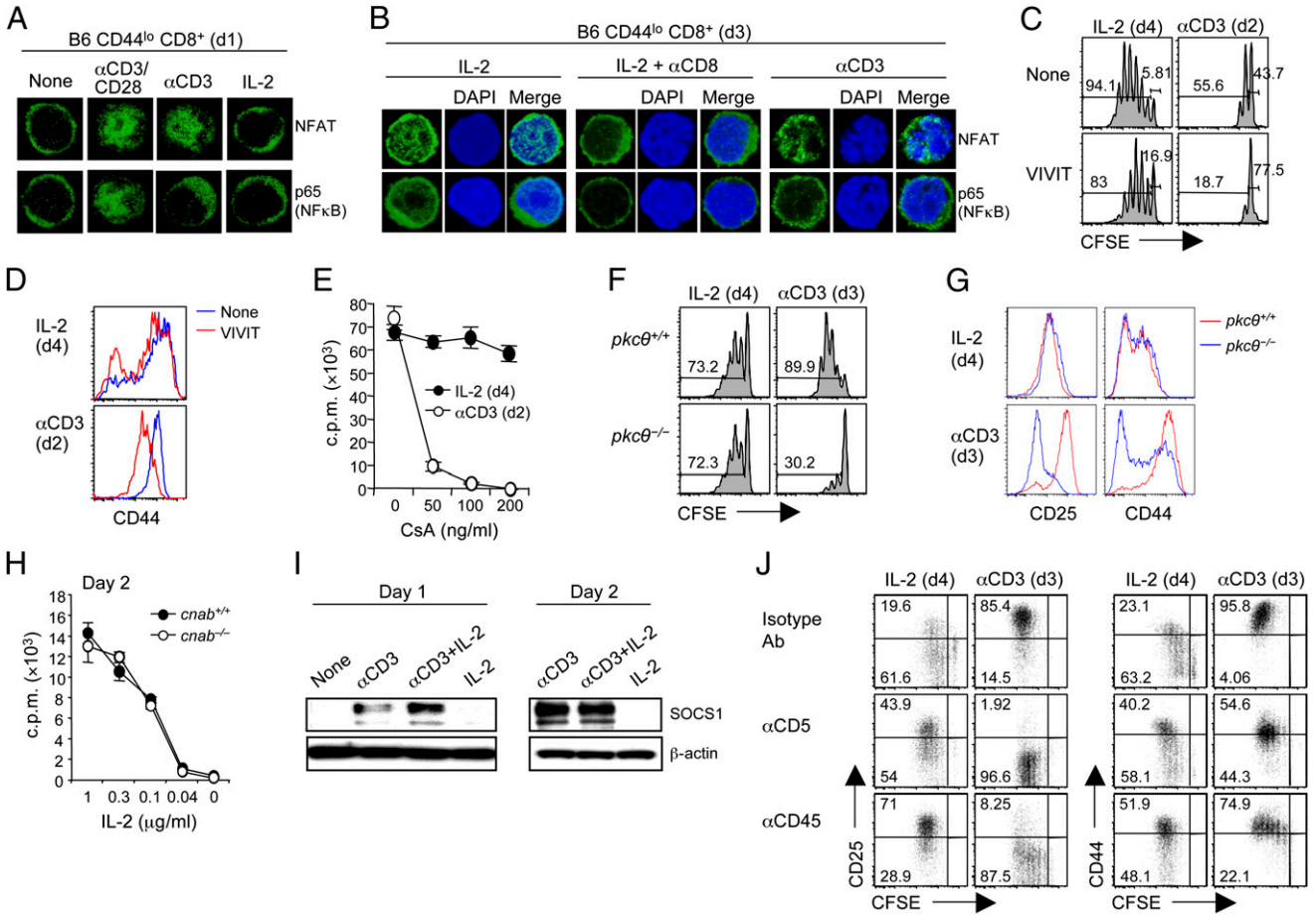

FIGURE 4. Role of NF-κB and NFAT in IL-2 signaling. Confocal staining (original magnification ×63) for nuclear translocation of NFAT and NF-κB (p65) after 1- (**A**) and 3-d (**B**) culture of naive B6 CD8 cells with indicated stimuli ± anti-CD8 blockade. CFSE division (**C**) and CD44 expression (**D**) and proliferation (**E**) after 2–4 d culture of naive B6 CD8 cells with IL-2 or plate-bound anti-CD3 ± VIVIT (C, D) and CsA (E). CFSE division (**F**) and CD25 and CD44 expression (**G**) after 3- to 4-d culture of naive WT and *pkcθ*$^{-/-}$ CD8 cells with IL-2 or plate-bound anti-CD3. (**H**) Proliferation after 2-d IL-2 exposure on naive WT and *cnaβ*$^{-/-}$ CD8 cells. (**I**) SOCS1 expression after 1- to 2-d culture of naive B6 CD8 cells with indicated stimuli. (**J**) CFSE division with CD25 and CD44 expression after 3- to 4-d culture of naive B6 CD8 cells with IL-2 or plate-bound anti-CD3 with anti-CD5, anti-CD45, or isotype control mAb (rat IgG). Percentages of gated region are shown. Data are representative of at least three independent experiments and shown as mean ± SD. α, Anti.

was completely abrogated by addition of anti-CD8 Ab, which impairs TCR/MHC-I interaction (Fig. 4B). This finding indicates that, as for proliferation (18), the modest nuclear translocation of NFAT induced by IL-2 in CD8 cells can be attributed to the low-level TCR signals resulting from recognition of self–MHC-I on neighboring T cells. Despite this finding, the calcineurin/NFAT pathway was not essential. Thus, in contrast to CD3 ligation, IL-2–induced proliferation and upregulation of activation markers on CD8 cells were not blocked by addition of VIVIT or CsA (Fig. 4C–E) and were unaffected in CD8 cells lacking calcineurin Aβ (*cnab*$^{-/-}$; Fig. 4H).

Together, the above findings indicate that, despite some overlap, the signaling pathways induced by IL-2 and TCR/CD3 ligation are distinctly different. In this study, along with the differences in signaling discussed above, synthesis of SOCS1 and SOCS3, a family of SOCS (32), was undetectable following IL-2 stimulation, though prominent with CD3 ligation (Fig. 4I, Supplemental Fig. 2). Interestingly, coligation of known negative regulators of TCR signaling, namely CD5 and CD45 (12, 32), accentuated the differences between IL-2 versus CD3 signaling. With CD3 ligation, coligation of CD8 cells with anti-CD5 or anti-CD45 Ab caused an appreciable reduction in the extent of proliferation and upregulation of activation markers (Fig. 4J). In marked contrast, CD5 or CD45 ligation of CD8 cells stimulated with IL-2 had the opposite effect and led to enhanced responses.

Expression of T-bet and Eomes

T cell responses to Ag involve synthesis of a number of differentiation-promoting transcription factors, such as Blimp-1, Bcl-6, T-bet, and Eomes (33, 34). For Blimp-1 and Bcl-6, these factors were expressed following either anti-CD3 or IL-2 stimulation, though more slowly with IL-2 than for CD3 ligation (Fig. 5A, 5B). The results for expression of T-bet and Eomes were diametrically opposite. Thus, CD3 or CD3/CD28 ligation induced rapid and persistent expression of T-bet but little or no expression of Eomes (Fig. 5A). By contrast, stimulation with IL-2 caused strong expression of Eomes, reaching a peak on 3 to 4 d, but induced barely detectable expression of T-bet, apparent only after prolonged exposure time (Fig. 5A, 5B). For CD8 cells cultured with IL-2, addition of IL-12, which is known to promote T-bet expression upon TCR stimulation (35), totally blocked IL-2–induced Eomes expression and instead led to T-bet expression (Fig. 5C); by contrast, addition of IL-21 had no effect, despite the known positive and negative influence of IL-21 on T-bet and Eomes, respectively, in CD4 and CD8 T cell differentiation (36, 37). Like IL-12, TCR ligation abolished the capacity of IL-2 to

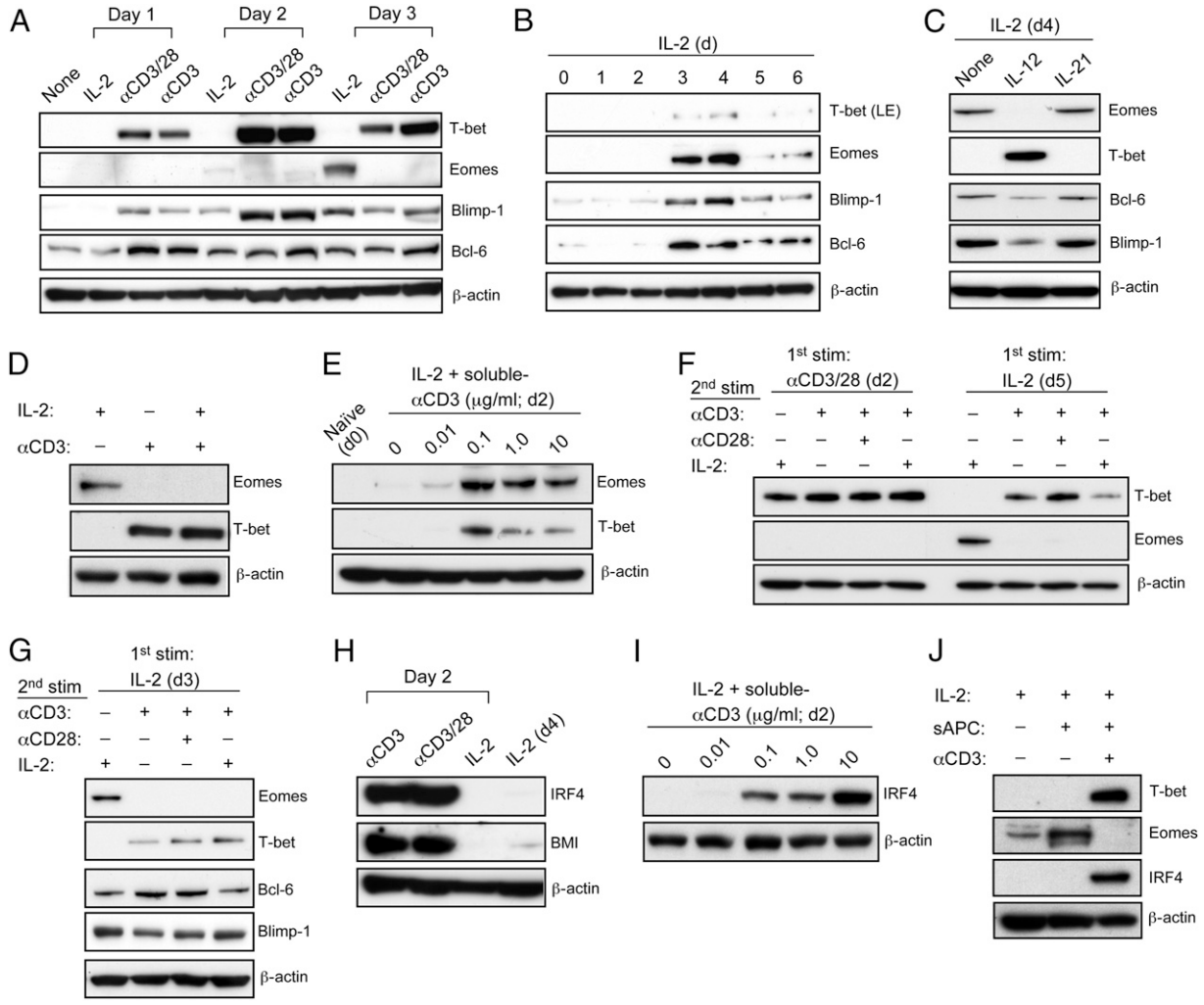

FIGURE 5. Regulation of T-bet and Eomes expression in vitro by IL-2. Expression of T-bet, Eomes, Blimp-1, and Bcl-6 after culturing naive B6 CD8 cells for indicated times with IL-2 or plate-bound anti-CD3 ± anti-CD28 (**A**), IL-2 alone (**B**), or IL-2 ± IL-12 or IL-21 (**C**). T-bet and Eomes expression after 2- to 3-d culture of naive B6 CD8 cells with IL-2, plate-bound anti-CD3 or both (**D**) and IL-2 with indicated amounts of soluble anti-CD3 (**E**). Fresh naive CD8 cells (day 0) were used as control (E). Expression of T-bet and Eomes (**F**, **G**) and Bcl-6 and Blimp-1 (G) in naive B6 CD8 cells preincubated with IL-2 (5 d, F; 3 d, G) or plate-bound anti-CD3/CD28 (2 d, F), followed by 3-d reculture with IL-2 or plate-bound anti-CD3 ± anti-CD28. Expression of IRF4 (**H**, **I**) and BMI (H) after 2–4 d culture of naive B6 CD8 cells with IL-2 or plate-bound anti-CD3 ± anti-CD28 (H) or IL-2 with indicated amounts of soluble anti-CD3 (I). (**J**) Expression of T-bet, Eomes, and IRF4 after 3-d culture of naive B6 CD8 cells with IL-2 ± sAPC ± soluble anti-CD3. Data are representative of two to three independent experiments. α, Anti.

induce Eomes expression, in parallel with strong T-bet induction (Fig. 5D). Notably, the capacity of TCR ligation to inhibit Eomes induction by IL-2 required strong TCR ligation (i.e., exposure to cross-linked rather than soluble anti-CD3 Ab). In fact, weak TCR ligation induced by soluble anti-CD3 Ab did not inhibit but instead enhanced IL-2–induced Eomes expression (Fig. 5E); T-bet expression increased in parallel, thus resulting in the combined synthesis of Eomes and T-bet.

The lack of T-bet expression after IL-2 stimulation was reversible because subsequent stimulation of the IL-2–cultured CD8 cells with cross-linked anti-CD3 or anti-CD3/CD28 Abs led to strong expression of T-bet, with concomitant loss of Eomes expression (Fig. 5F); there was no change in Bcl-6 and Blimp-1 (Fig. 5G). By contrast, the failure of CD3 ligation to induce Eomes seemed irreversible, as Eomes remained undetectable when the CD3/CD28-prestimulated cells were subsequently cultured with IL-2 alone (Fig. 5F).

For other transcription factors, IL-2 stimulation differed from CD3 ligation in being unable to induce expression of IRF4 or BMI-1 (Fig. 5H) (i.e., factors crucial for CD8 T cell differentiation) (38, 39). Hence, like T-bet but unlike Eomes, expression of these two factors was heavily dependent on TCR signals. However, as for T-bet, the failure of IL-2 to induce IRF4 could be overcome by addition of soluble anti-CD3 Ab (Fig. 5I), though, interestingly, not by addition of normal sAPC (Fig. 5J) (i.e., cells presenting normal self-MHC ligands). In this latter situation, addition of sAPC plus IL-2 amplified Eomes induction but failed to cause IRF4 synthesis (Fig. 5J). These findings refer to cells cultured in vitro. The effects of IL-2 on the expression of T-bet and Eomes under in vivo conditions are considered later.

Differentiation into polarized effector cells

Although culturing naive CD8 cells with IL-2 alone induced extensive proliferation and upregulation of various activation markers, generation of effector function was limited. Thus, correlating with the lack of T-bet expression, IL-2–stimulated CD8 cells, unlike CD3-ligated cells, were unable to produce IFN-γ unless briefly restimulated with anti-CD3 Ab (Fig. 6A, 6B). However, stimulation of T-bet in the cells by adding a mixture of

IL-2 plus soluble anti-CD3 Ab or IL-2 plus IL-12 led to more extensive proliferation, higher expression of activation markers (CD25 and CD44), and enhanced synthesis of IFN-γ and granzyme B (Fig. 6A, 6C–E); similar though less marked effects were seen with a mixture of IL-2 and IL-21.

Confirming previous work, subjecting naive CD4 or CD8 cells to CD3/CD28 ligation under typical Th1-polarizing conditions (IL-12 plus anti–IL-4) led to strong synthesis of Th1 cytokines, notably IFN-γ (Fig. 6F); however, for CD8 cells, IFN-γ synthesis with Th1-polarizing conditions was no higher than with CD3/CD28 ligation without polarizing cytokines (Th0). Conversely, culture under Th2 conditions (IL-4 plus anti–IFN-γ) produced the canonical Th2 cytokines (IL-4, IL-6, and IL-10). Very different results applied for stimulation via IL-2. In this study, culturing naive CD8 cells under Th1-polarizing conditions paradoxically led to strong production of Th2 cytokines, notably IL-6 and IL-10, both of which were produced by CD3/CD28 stimulation of CD8 (and CD4) cells under Th2-polarizing conditions (Fig. 6G); indeed, except for IL-4 synthesis, fold increase in CD8 cell production of Th2 cytokines after IL-2 stimulation (relative to the levels induced under Th0 conditions) was higher under Th1 than Th2 conditions (Fig. 6F). These findings are highly surprising and difficult to explain, particularly because IL-12 (used for Th1 polarization) led to strong T-bet expression (Fig. 5C).

Survival and function in vivo

The high induction of T-bet induced by strong TCR/CD3 signaling is known to favor generation of short-lived effector CD8 T cells but curtail their survival as memory cells (40, 41). Because T-bet expression was limited and transient in naive CD8 cells stimulated with IL-2 alone in vitro, these cells might survive well after transfer in vivo.

To investigate this idea, naive CD8 cells from 2C or OT-I TCR-transgenic mice were cultured for 5 d with IL-2 alone in vitro and then transferred to B6 mice. One week after transfer, the donor 2C cells displayed potent in vivo cytotoxic activity against syngeneic target splenocytes (Ly5.1) pulsed with specific peptide (SIYRp; Fig. 7A). Lysis was comparable to that of control CD3-stimulated cells, and there was no lysis of unpulsed cells. In further studies, we examined responses to *L. monocytogenes*, an established model for testing CD8 T cell effector function in vivo. In this study, similar potent effector function soon after transfer was displayed by IL-2–stimulated OT-1 cells, as defined by responses to Lm-ova, the ligand for OT-1 cells (Fig. 7B, 7C); Lm expressing an irrelevant Ag (Lm-gp33) was used as a control. Injection of Lm-ova led to marked Ag-specific expansion of the transferred IL-2–stimulated OT-1 cells followed by rapid elimination of bacterial counts in the spleen (Fig. 7B, 7C) as well as prolonged survival of the hosts after injection of lethal doses of Lm-ova infection (Fig. 7D). The protection afforded by the IL-2–stimulated cells was as marked as with CD3/CD28-stimulated cells (Fig. 7D). Transfer of naive unstimulated OT-1 cells gave minimal expansion and protection (Fig. 7B–D), indicating that prior contact with IL-2 in vitro greatly enhanced the effector function of the cells after transfer in vivo. Collectively, these findings indicated that, despite their limited expression of effector function in vitro, IL-2–stimulated CD8 cells displayed strong effector function after adoptive transfer in vivo.

To assess long-term in vivo survival of IL-2–stimulated cells, 2C CD8 cells were cultured in vitro for 5 d with IL-2 and then examined at various periods after transfer to normal B6 mice. The transferred 2C cells did not undergo noticeable in vivo expansion

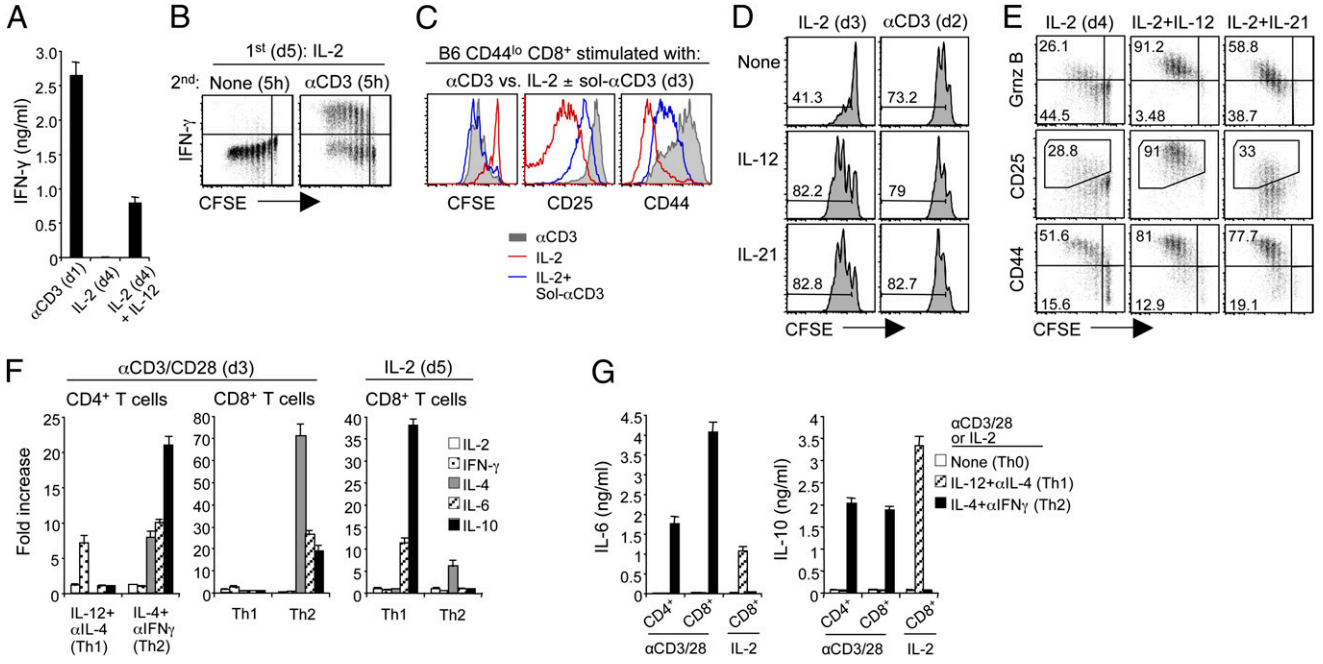

FIGURE 6. Effector generation and cytokine polarization by IL-2. (**A**) IFN-γ ELISA after 1–4-d culture of naive B6 CD8 cells with plate-bound anti-CD3 or IL-2 ± IL-12. (**B**) CFSE division and IFN-γ synthesis in naive B6 CD8 cells precultured with IL-2 (5 d), followed by 5-h reculture ± plate-bound anti-CD3. (**C**) CFSE division (*left panel*) and CD25 and CD44 expression (*middle and right panels*) after 3-d culture of naive B6 CD8 cells with plate-bound anti-CD3 or IL-2 ± soluble anti-CD3. CFSE division (**D**, **E**) and expression of granzyme B (Grnz B), CD25, and CD44 (E) after 2–4-d culture of naive B6 CD8 cells with plate-bound anti-CD3 (D), or IL-2 ± IL-12, or IL-21 (D, E). (**F**) Culture supernatants from naive B6 CD4 and CD8 cells prestimulated with IL-2 (5 d) or plate-bound anti-CD3/CD28 (3 d) ± indicated Th1-, Th2-, or Th0-polarizing condition were analyzed for the indicated cytokines by ELISA. Fold increase of each cytokine produced in Th1 or Th2 conditions relative to Th0 condition is shown. (**G**) IL-6 and IL-10 production from indicated CD4 and CD8 cells cultured as in (F). Percentages of gated region are shown. Data are representative of three independent experiments and shown as mean ± SD. α, Anti.

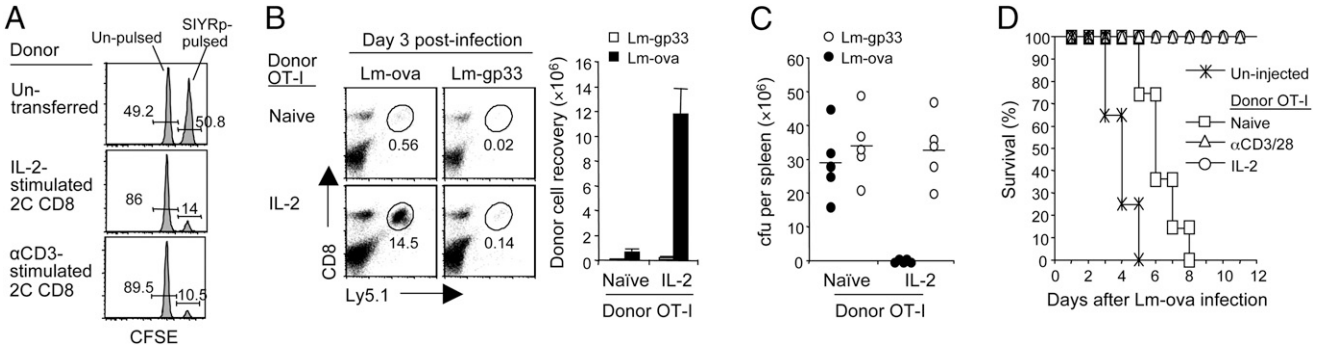

FIGURE 7. In vivo effector function. (**A**) In vivo CTL activity to SIYRp-pulsed ($CFSE^{hi}$) and unpulsed ($CFSE^{lo}$) splenocytes transferred to B6 recipient mice either uninjected or injected with indicated 2C donor cells 7 d before ($n = 3$). (**B** and **C**) Naive or IL-2–stimulated OT-I cells (Ly5.1) were injected into B6 mice, and 1 d later, the mice were infected with Lm-ova or Lm-gp33 ($n = 5$). Expansion (B, *left panel*) and recovery of OT-I donor cells (B, *right panel*) and bacterial counts (C) in spleen of the mice were analyzed at 3 d postinfection. (**D**) Survival rate after Lm-ova infection to B6 recipient mice either uninjected or injected with freshly isolated naive, IL-2–, or anti-CD3/CD28–stimulated OT-I cells 3 d before ($n = 6$–8). Percentages of gated region are shown. Data are representative of two to three independent experiments and shown as mean ± SD. α, Anti.

but rather persisted for >2 mo (62 d) with constant donor cell recoveries in spleen and LNs (Fig. 8A). To compare the long-term survival of IL-2–stimulated cells with TCR-stimulated cells, naive OT-I cells were cultured in vitro with IL-2 or cross-linked anti-CD3/CD28 Abs and then injected in comparable numbers to normal B6 hosts (Fig. 8B); in some cases, anti-CD3 Ab plus IL-2 was used for in vitro TCR stimulation (Fig. 8C). When examined in PBLs, the donor-derived TCR-stimulated OT-I cells were prominent at 3 d posttransfer but then declined rapidly in number (Fig. 8B), consistent with rapid elimination of $T\text{-bet}^{hi}$ CD8 effector cells (41, 42). With transfer of IL-2–stimulated OT-I cells, by contrast, donor cell recoveries were low on 3 d but then remained relatively constant for prolonged periods (Fig. 8B). At 1–6 mo posttransfer, total recoveries of the IL-2–stimulated OT-I cells in PBL, spleen, and LN were one-fourth to one-half the level found for TCR-stimulated OT-I cells (Fig. 8B, 8C). Throughout this period, IL-2–stimulated 2C or OT-I donor populations showed the typical phenotype of central memory cells (i.e., high expression of CD44, CD122, CD62L, and CD127) (Fig. 8D, 8E) and displayed strong effector functions, such as in vivo CTL activity (Fig. 8F, 8G), and synthesis of effector cytokines TNF-α and IFN-γ, after exposure to specific SIYRp or OVAp Ags in vitro (Fig. 8H, 8I).

Because T-bet and Eomes are known to influence the generation and survival of memory CD8 T cells in vivo (43), it was important to examine the effects of IL-2 stimulation under in vivo conditions. In initial studies on normal B6 mice, background expression of both T-bet and Eomes in CD8 cells was clearly higher in resting MP $CD44^{hi}$ cells than in naive $CD44^{lo}$ cells (Fig. 9A), which correlated with higher expression of CD122 ($CD122^{hi}$) on MP cells and the known role of Eomes in inducing CD122 expression (43). For T-bet, similar findings applied after transfer of in vitro–stimulated OT-I cells. Thus, both for cells cultured with IL-2 alone or with anti-CD3 Ab (plus IL-2), the long-term (56 d) progeny of these cells after adoptive transfer showed the same $T\text{-bet}^{hi}$ $CD44^{hi}$ phenotype as host CD8 MP cells (Fig. 9B); with transfer of naive OT-1 cells, by contrast, T-bet levels remained low. Different findings applied to Eomes expression. With transfer of CD3-stimulated OT-1 cells, Eomes expression in these cells was relatively low within the first few weeks after transfer (32 d; Supplemental Fig. 3A) and then gradually increased, though Eomes expression was less than in host MP cells even at 2 to 3 mo after transfer (Fig. 9B, Supplemental Fig. 3B). With transfer of IL-2–stimulated cells, by contrast, Eomes expression was high soon after transfer and remained high thereafter (Fig. 9B, Supplemental Fig. 3A), consistent with the rapid conversion of IL-2–stimulated cells into central memory cells.

To examine IL-2 stimulation under in vivo conditions, naive 2C cells were transferred into irradiated B6 hosts and then stimulated in situ by injection of IL-2 (using IL-2/anti–IL-2 complexes) versus specific SIYRp. In uninjected control mice, the lymphopenic environment of the irradiated hosts caused the donor 2C cells to proliferate briefly and differentiate into typical $CD44^{hi}$ MP cells, presumably driven by the raised levels of IL-7 in these hosts (44). During the proliferative stage soon after transfer (6 d), the donor 2C cells showed low but significant upregulation of both T-bet and Eomes, relative to control naive 2C cells transferred into normal B6 hosts (Fig. 9C, *top panel*); thereafter (42 d), T-bet expression remained stable, whereas Eomes expression further increased. Essentially similar findings, though with stronger induction of Eomes, occurred after injection of IL-2 (Fig. 9C, *middle panel*). As with stimulation by IL-7 or IL-2, in vivo TCR stimulation by the injection of SIYRp induced rapid and persistent upregulation of T-bet (Fig. 9C, *bottom panel*). In marked contrast, induction of Eomes by SIYRp was conspicuously slow, being almost undetectable at 6 d and only moderately elevated even after 6 wk (42 d).

These data indicate that, as in vitro, Eomes induction in CD8 cells was rapid and prominent with stimulation via cytokines (IL-7 and/or IL-2) but slow and limited with a TCR stimulus. Significantly, as in vitro, strong TCR signaling inhibited Eomes induction by IL-2 in vivo. Thus, for OT-1 cells, the prominent induction of Eomes but not T-bet induced by injection of IL-2 was reversed when a mixture of IL-2 and SIYRp was injected: adding SIYRp reduced Eomes expression but increased T-bet and also granzyme B synthesis (Fig. 9D).

For T-bet expression, the prominent CD8 T cell induction of T-bet elicited by SIYRp in vivo correlated well with the results of strong TCR ligation in vitro. For stimulation via IL-2, however, the results were different. Thus, contact with IL-2 (or IL-7) caused low but significant T-bet induction only in vivo (Fig. 9C) and not in vitro (compare Fig. 9C with Fig. 5D). Because the lack of T-bet induction by IL-2 in vitro could be overcome by adding a very weak TCR stimulus (soluble anti-CD3 Ab), T-bet induction by IL-2 in vivo might reflect low-level TCR signals arising from constant contact with self–MHC-I ligands. In support of this idea, induction of T-bet expression on CD8 cells in vivo was minimal when these cells were stimulated with or without cytokines in situ after transfer to irradiated Tap1-deficient hosts (Fig. 9E, Supplemental Fig. 3C).

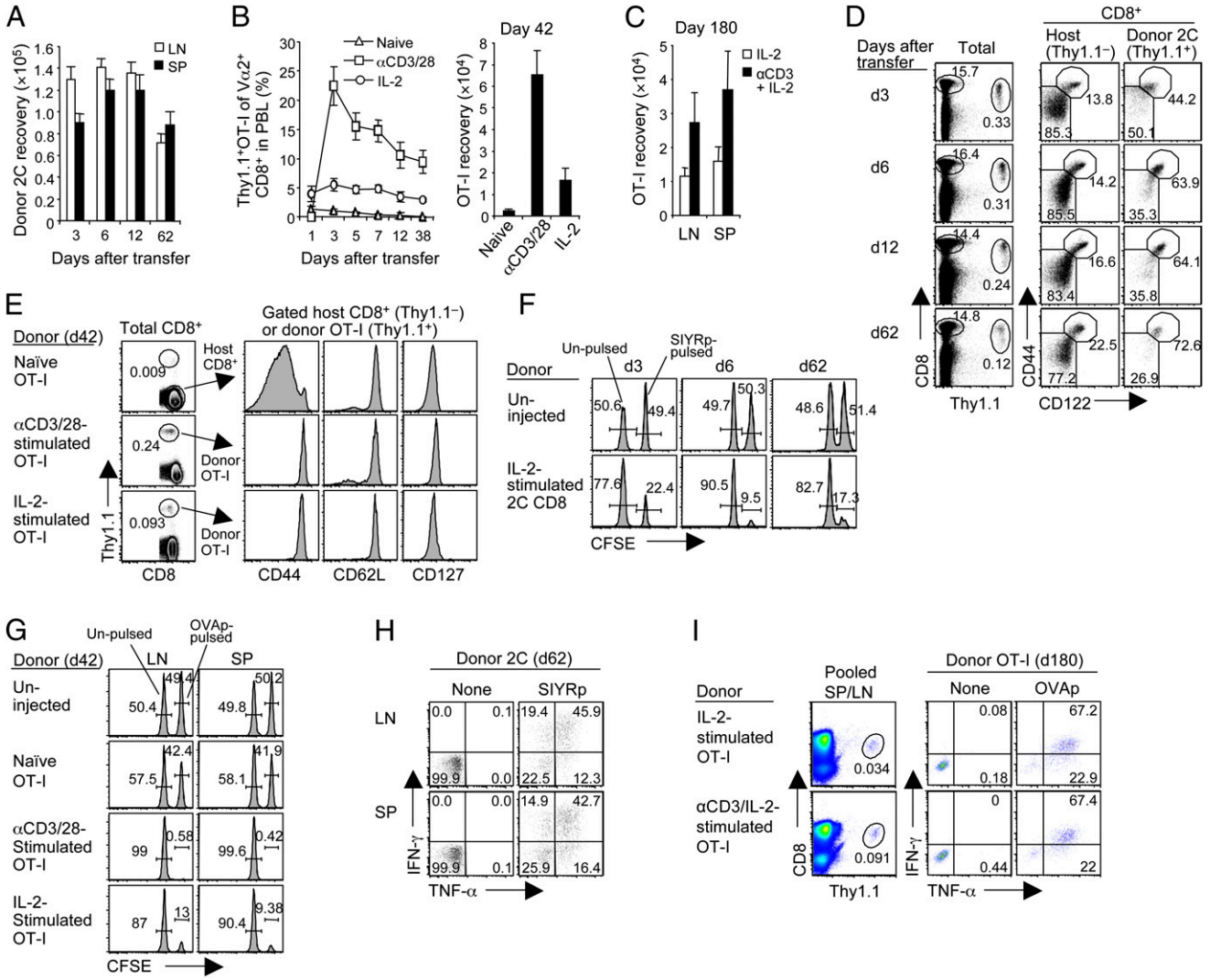

FIGURE 8. Memory cell differentiation. (**A**) Naive or IL-2–stimulated 2C cells (Thy1.1) were injected into B6 mice. Donor cell recovery in LN and spleen (SP) of the mice was analyzed at indicated times (n = 3–5). Naive, IL-2– (**B**, **C**), anti-CD3/CD28– (B), or anti-CD3/IL-2–stimulated (C) OT-I cells (Thy1.1) were injected into B6 mice. Recovery of OT-I donor cells in PBL (B; *left panel*), pooled spleen/LN (B; *right panel*) or in spleen (SP) and LN (C) was analyzed at indicated times (n = 4 to 5). (**D**) CD44 and CD122 expression on gated host CD8 and donor 2C cells from spleen of the mice in (A). (**E**) Expression of indicated surface markers on gated host CD8 and donor OT-I cells from pooled spleen/LN of the mice in (B) (42 d). In vivo CTL activity from the recipient mice in (A) (**F**, 3, 6, and 62 d; n = 2 to 3) or in (B) (**G**, 42 d; n = 2) using transfer of CFSE-labeled SIYRp- (F) or OVAp-pulsed (G) target cells. IFN-γ and TNF-α synthesis on gated 2C (**H**) or OT-I (**I**) donor cells from the recipient mice in (A) (62 d) and (C) (180 d), respectively, after 5-h restimulation with SIYRp (H) or OVAp (I). Percentages of gated region are shown. Data are representative of two to three independent experiments and shown as mean ± SD. α, Anti.

Discussion

Because TCR signaling leads to rapid synthesis of IL-2 by the responding cells, the relative importance of TCR versus IL-2R signaling in T cell activation and proliferation is difficult to define, especially for normal naive T cells. For IL-2 signaling, previous studies demonstrated that purified naive CD8 T cells can proliferate in response to IL-2 in the absence of Ag (17, 18, 45). In the current study, using highly purified normal T cells in the absence of other cells, we show that naive CD8 cell signaling induced by IL-2 is distinct and largely different from the pathways involved in TCR stimulation.

In the case of downstream signaling, in accordance with previous studies on murine and human T cell lines (46–49), IL-2–induced proliferation of naive CD8 T cells in vitro was heavily dependent on signaling via the PI3K/AKT/mTOR pathways; thus, inhibition of these pathways ablated CD8 T cell proliferation. By contrast, the MAPK pathways were relatively unimportant in IL-2 signaling: IL-2 elicited phosphorylation of ERK, JNK, and p38, but adding inhibitors of these MAPKs only marginally impaired IL-2–induced proliferation of CD8 cells. Likewise, IL-2–induced proliferation did not involve the NF-κB or NFAT pathways. For NF-κB, IL-2 failed to cause nuclear translocation of NFκB1 p65, and proliferation to IL-2 was unimpaired in CD8 cells lacking upstream PKCθ. For NFAT, although IL-2 did cause late-onset nuclear translocation of NFAT, IL-2–induced proliferation was not reduced by addition of the NFAT inhibitors, VIVIT, or CsA. These findings with IL-2 contrasted with the opposite results seen with TCR ligation. In this study, proliferation induced by CD3 or CD3/CD28 ligation was easily inhibited by blocking ERK, PKCθ, or the Ca^{2+}/calcineurin/NFAT pathway but less easily inhibited by suppressing the PI3K/AKT/mTOR pathway.

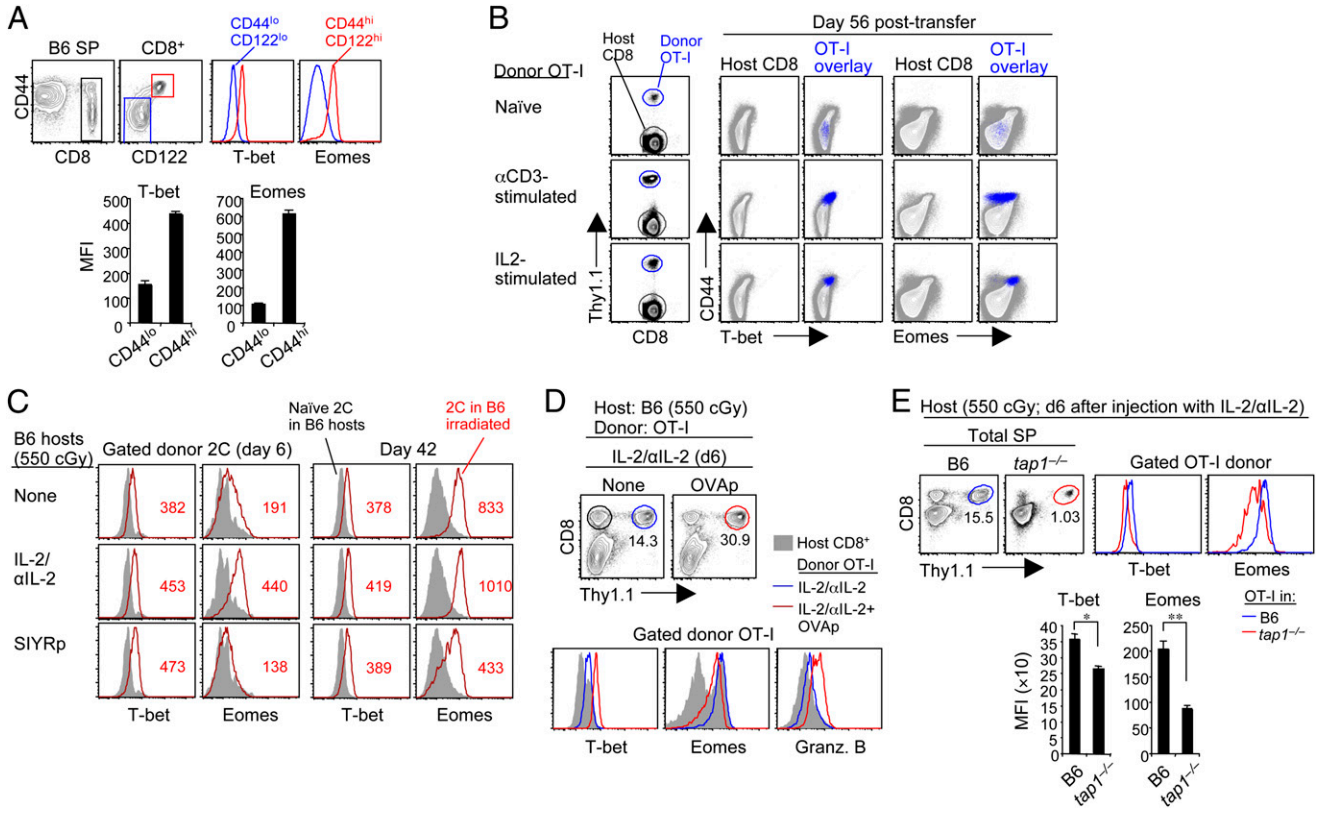

FIGURE 9. In vivo regulation of T-bet and Eomes expression by IL-2. (**A**) FACS plots (*top panel*) and mean fluorescence intensity (MFI; *bottom panel*; $n = 5$) for T-bet and Eomes expression on indicated gated CD8 cells from SP of normal B6 mice. (**B**) T-bet and Eomes expression on gated host CD8 and donor OT-I cells from spleen of B6 mice ($n = 4$ to 5) injected with naive, IL-2–, or anti-CD3/IL-2–stimulated OT-I cells 56 d before. (**C**) T-bet and Eomes expression (MFI included) on gated donor 2C cells from spleen of irradiated B6 mice ($n = 5$) injected with naive 2C cells ± IL-2/anti–IL-2 complexes or SIYRp 6 and 42 d before. (**D**) T-bet and Eomes expression on gated host and donor OT-I cells from spleen of irradiated B6 mice ($n = 3$) injected with naive OT-I cells plus IL-2/anti–IL-2 complexes ± OVAp 6 d before. (**E**) FACS plot (*top panel*) and MFI (*bottom panel*) for T-bet and Eomes expression on gated donor OT-I cells from spleen of irradiated B6 and *tap1*$^{-/-}$ mice ($n = 3$ to 4) injected with naive OT-I cells plus IL-2/anti–IL-2 complexes 6 d before. Percentages of the gated region are shown. Data are representative of at least two independent experiments and shown as mean ± SD. *$p < 0.001$, **$p < 0.0001$. α, Anti.

As expected from previous studies on IL-2R signaling with cell lines (50–53), stimulation of naive CD8 cells by IL-2 depended crucially on JAK3. Thus, IL-2 elicited rapid phosphorylation of JAK3, and chemical inhibition of JAK3 abolished proliferation; likewise, IL-2–induced proliferation was far lower with *jak3*$^{+/-}$ cells than with *jak3*$^{+/+}$ cells. By contrast, CD3-induced proliferation was relatively resistant to JAK3 blockade. Significantly, IL-2 signaling via JAK3 was heavily dependent on LCK. This finding is of interest because prior studies on the role of LCK in IL-2 signaling are conflicting. Thus, although initial studies on cell lines showed LCK association with IL-2Rβ (30) and LCK-dependent induction of c-Fos and c-Jun after IL-2 stimulation (54), subsequent experiments failed to implicate LCK in IL-2–induced proliferation (55, 56). As shown in this study, however, culturing naive CD8 cells with IL-2 in the presence of an LCK inhibitor abolished IL-2–induced proliferation; such inhibition was associated with lack of JAK3 phosphorylation but retention of JAK1 phosphorylation. Together, these findings indicate that, for naive CD8 cells, IL-2/IL-2R signaling leads to proliferation via a pathway involving sequential phosphorylation of LCK, then JAK3 followed by PI3K/AKT/mTOR and other downstream substrates (Fig. 3O). Why LCK is needed for IL-2 stimulation of naive CD8 cells but not cell lines is unclear, although differences in the distribution of signaling proteins in lipid rafts could be a factor (18).

Surprisingly, in marked contrast to JAK3, STAT5 function seemed to be largely irrelevant for IL-2–induced proliferation. Consistent with reports on T cell lines (57, 58), culturing naive CD8 cells with IL-2 induced rapid STAT5 phosphorylation. Nevertheless, short-term (3 d) proliferative responses to IL-2 were as high with *stat5*$^{-/-}$ cells as with WT cells. Later (5 d) responses were partly reduced with *stat5*$^{-/-}$ cells, which suggests a role for STAT5 in cell survival rather than proliferation. However, further studies are needed to clarify this issue because the *stat5*$^{-/-}$ mice used may contain a partially functional N-terminally truncated STAT5 polypeptides (59, 60). It is of interest that, based on LCK blocking studies and the use of *jak3*$^{+/-}$ cells, STAT5 phosphorylation by IL-2 showed little or no requirement for either JAK3 or LCK. Hence, STAT5 phosphorylation seemed to be the direct result of IL-2R ligation. In this study, there was a contrast with ERK phosphorylation that, though JAK3 independent, was strongly dependent on LCK, perhaps via Shc. Like STAT5 phosphorylation, IL-2–induced ERK phosphorylation might play a role in cell survival and/or later stages of the immune response (Fig. 3O). As discussed above, however, IL-2–induced proliferation seemed to be largely independent of both ERK and STAT5 but instead depended heavily on LCK/JAK3-dependent signaling via the PI3K/AKT/mTOR pathway.

With regard to generation of effector function, it is well documented that typical TCR-driven responses to Ag are associated with rapid synthesis of T-bet, which is essential for the formation of typical Th1 responses and IFN-γ synthesis (35). In this respect, it is striking that, despite extensive proliferation, stimulation of na-

ive CD8 cells with IL-2 caused barely detectable T-bet expression and a failure to synthesize IFN-γ unless the cells were restimulated by TCR ligation. By contrast, supplementing IL-2 with IL-12 led to strong induction of T-bet and prominent IFN-γ synthesis. These findings are consistent with the notion that IFN-γ production depends crucially on T-bet induction and vice versa (61), and also that IFN-γ and T-bet synthesis both depend on NF-κB activation (62), the latter being undetectable with IL-2 stimulation. Nevertheless, it is surprising that, unlike IL-12 plus IL-2, a mixture of IFN-γ plus IL-2 failed to induce T-bet synthesis by CD8 cells but instead augmented Eomes (Supplemental Fig. 4A). It is also surprising that culturing CD8 cells with IL-2 under Th1-polarizing conditions led to strong production of Th2 cytokines. This finding is difficult to explain because the presence of IL-12 in the Th1-polarized cultures presumably induced T-bet, which, at least in CD4 cells, has been found to suppress Th2 cell development (63). However, these data on IL-2 stimulation nevertheless seem to fit with the long-standing view that lack of strong TCR signaling favors Th2 rather than Th1 development (64, 65).

With CD3 ligation of CD8 cells, rapid synthesis of T-bet was accompanied by a complete lack of Eomes synthesis, even when the cultures were supplemented with various combinations of cytokines (Supplemental Fig. 4B). With stimulation of CD8 cells via IL-2, by contrast, failure to induce T-bet expression was balanced by relatively rapid (3 d) induction of Eomes. This finding was unexpected because previous studies showed that Eomes was expressed quite slowly in culture, even when preactivated CD8 cells were cultured with IL-2 for extended periods (66, 67). For naive CD8 cells, our finding that addition of IL-12 or CD3 ligation abrogated Eomes induction by IL-2 implies that normal T cell activation via TCR signaling suppresses Eomes induction. Such suppression could reflect TCR-mediated induction of IRF4 because TCR/ITK-dependent generation of IRF4 is known to inhibit Eomes expression in CD8 cells (68). Surprisingly, however, we observed that weak TCR signaling provided by soluble anti-CD3 Ab plus IL-2 induced IRF4 synthesis yet paradoxically caused an increase, not a decrease, in Eomes expression. Why only strong and not weak TCR signaling inhibits Eomes expression is still unclear. Recently, it was reported that Foxo1 represses T-bet induction while enhancing Eomes expression (69). With IL-2 stimulation, however, we have found that IL-2 induces strong phosphorylation of Foxo1 (Supplemental Fig. 4C), thereby presumably ablating its activity. Hence, Eomes induction by IL-2 may be Foxo1 independent.

The opposing effects of strong versus weak TCR signaling on Eomes expression may explain why CD8 cells cultured with IL-2 versus CD3 ligation displayed different fates after adoptive transfer. With transfer of CD3-stimulated CD8 cells, most of the cells died within 1 to 2 wk after transfer, and only a small proportion of the cells survived to form long-lived memory cells; these findings are closely in line with the observation that typical immune responses of T cells in vivo are associated with conspicuous cell death at the end of the response (70). The fate of IL-2–stimulated cells was quite different. Surprisingly, despite only weak effector function in vitro, IL-2–stimulated CD8 cells displayed potent effector function soon after transfer in vivo, possibly through exposure to T-bet–inducing cytokines such as IL-12 in the in vivo environment. The striking finding with IL-2–stimulated CD8 cells, however, was that attrition of the cells after transfer was very limited, and the cells differentiated rapidly into functionally competent typical central memory cells.

Significantly, the survival of IL-2–stimulated CD8 cells without a contraction phase prior to their differentiation into memory cells was associated with continuous strong expression of Eomes and low expression of T-bet. By contrast, the progressive death of the CD3-stimulated cells after transfer was associated with prominent T-bet expression but only very limited expression of Eomes. These differences in Eomes versus T-bet expression were also seen when CD8 cells were stimulated with specific peptide versus IL-2 under in vivo conditions. It is of interest that the lack of a contraction phase seen in vivo after IL-2 stimulation has also been reported for CD8 cells stimulated with Ag plus rapamycin (71), the latter being known to repress T-bet expression while enhancing Eomes expression.

The data fit well with the view that T-bet expression favors the generation of short-lived effector T cells, whereas Eomes inhibits death and guides the generation and survival of memory cells (43, 72). Notably, expression of Eomes and T-bet remained very low when CD8 T cells were stimulated with IL-2 in situ after transfer to irradiated *tap1*$^{-/-}$ hosts. Hence, the combined expression of both Eomes and T-bet in long-lived memory CD8 cells, as well as the component of MP cells found in normal mice, presumably reflects continuous low-level TCR signaling resulting from TCR/self–MHC-I interaction, though the effects of such signaling are subtle in vitro.

With regard to physiological significance, stimulation of CD8 cells by IL-2 under normal conditions in vivo is probably largely restricted to Ag-stimulated naive CD8 cells exposed to IL-2 secreted either by the responding cells or by adjacent CD4 helper cells. Such combined TCR plus IL-2–induced signaling presumably involves the multiple pathways described in this study and in previous publications and is also likely to apply during IL-2 therapy for treatment of cancer. For Ag-independent stimulation by IL-2, the high concentrations of cytokines (0.1–1 μg/ml) needed to induce proliferation of purified naive CD8 cells in vitro suggests that bystander stimulation of naive CD8 cells by IL-2 under normal in vivo conditions is quite limited. As mentioned earlier, however, contacts with related γ_c cytokines, namely IL-7 and IL-15, play an important role in naive CD8 cell homeostasis and, like IL-2, can induce marked proliferation of these cells when cytokine levels are raised (e.g., in lymphopenic or IL-2/15R–deficient hosts and also after injection of these cytokines) (17, 73). For the latter, based on our results for IL-2, the success of γ_c cytokine therapy for restoring T cell numbers in conditions of lymphopenia (74, 75) could be due in large part to strong cytokine-mediated induction of Eomes with minimal T-bet expression.

Acknowledgments

We thank C. King and K. Webster for reading the manuscript and comments, A. Hong for mice genotyping, R. Salomon and D. Snowden for cell sorting, the Pieter Huveneers Molecular Imaging Unit (Garvan Institute of Medical Research) for confocal imaging, and the Biological Testing Facility (Garvan Institute of Medical Research) for animal care.

Disclosures

The authors have no financial conflicts of interest.

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J Immunol 2013; 191:5559-5573; Prepublished online 28 October 2013;
doi: 10.4049/jimmunol.1302293
http://www.jimmunol.org/content/191/11/5559

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.